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Inspectors Instructions for Aflatoxin

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This instruction is designed for Processed Products Branch personnel of the U.S. Department of Agriculture. Its purpose is to present an analytical method for the determination of aflatoxin in peanuts, peanut products and other miscellaneous products. It is intended for the individual who is familiar with aflatoxin methodology and the equipment necessary for the performance of the work. A knowledge of chemistry is required to insure accurate and safe determination of aflatoxin levels. The analyst is cautioned that aflatoxin is a known toxin to animals, a known animal carcinogen and a suspected human carcinogen.

This instruction utilizes official methods of analysis of the Association of Official Analytical Chemists (AOAC) with modifications for a larger sample size. Other information used in this instruction was obtained from sources available to the public as well as technical knowledge of personnel in the Department.

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I. HISTORY

Plants, animals, and micro-organisms can be sources of naturally occurring toxic substances which may remain in foods unless adequate precautions are taken to eliminate them. Many of the naturally occurring toxic components of food have received comparatively little attention, but acute (short term) and chronic (long term) toxicity have been recognized as results of man and animals eating foods contaminated with these substances.

Micro-organisms (molds, yeasts, and bacteria) are known to produce toxic metabolites called microbial toxins. In considering microbial toxins the scientist is not concerned with the infectious nature of the micro-organism but with the toxic metabolites produced when the micro-organisms grow on foods. Examples of this class of toxic materials are the botulinum and staphylococcus toxins produced by bacteria. Less familiar are the mycotoxins produced by molds, such as the aflatoxins, the ochratoxins, and the extrogenic substances.

Mycotoxins have undoubtedly been with man since the beginning. However, they were not recognized as health problems until relatively recent years. The current emphasis in this field was stimulated in 1961 when a large number of turkeys died in England. British researchers investigated the "Turkey X" disease and found that the causative toxic material was a peanut meal coming from Brazil. They further related the toxic principle to the mold Aspergillus flavus and coined the word aflatoxin.

It is now known that the aflatoxins are also produced by the mold Aspergillus parasiticus, in addition to Aspergillus flavus. These are very potent toxins for some animals and the sensitivity varies over a considerable range for different species. Rainbow trout are the most sensitive animals that have been found so far. It is interesting to note that brown trout are relatively resistant compared to rainbows. Ducklings are also quite sensitive, whereas sheep are the most resistant animals that have been studied. In addition to the acute toxicity of the aflatoxins, they have been found to be carcinogens for some animals.

Human sensitivity to aflatoxins is not presently known, however it is suspected that there are toxic effects to humans from aflatoxin. The Food and Drug Administration (FDA) has recognized this probable toxic effect in setting an administrative guideline of 20 ppb aflatoxin in foods suspected to be susceptible to aflatoxin contamination, with the exception of milk, meat, and eggs which have an enforcement level of 0.5 ppb aflatoxin. Animal feeds also are subject to the 20 ppb guideline. Raw shelled peanuts have a tolerance of 25 ppb aflatoxin since it is recognized that sorting and roasting the peanuts will reduce the aflatoxin content sufficiently to meet the FDA administrative guideline of 20 ppb aflatoxin in finished peanut products.

II. SAMPLING

The first prerequisite for getting information on a lot of merchandise is obtaining a representative sample.

A. Shelled Peanuts

Shelled peanuts are sampled for testing for the Peanut Administrative Committee (PAC) in accordance with the PAC sampling plan (1). The maximum lot size permitted is 200,000 lbs. Sampling of peanuts for aflatoxin is extremely difficult because of non-uniformity of the contaminant in the lot.

Lot sampling -- Shelled peanuts are sampled in such a manner as to make possible a sequential testing and acceptance procedure. Sufficient peanuts are sampled from each lot by the Federal-State Inspection Service to provide three 48-pound samples designated by the inspector as Sample No. 1, Sample No. 2, and Sample No. 3. Each sample is placed in a suitable container and "positive lot identified." Sample No. 1 is ground in a "Dickens Subsampling Mill." The amount from the subsample output should be 1100 grams ±100 grams and this shall be designated as "Subsample 1 AB." Samples No. 2 and 3 are held in reserve for possible analysis under the sequential testing and acceptance procedure approved by PAC. Subsample 1 AB is for immediate analysis.

B. Peanut Butter

Peanut Butter is a homogeneous product. Therefore, obtaining a representative sample is relatively easy.

In-Plant Samples -- Samples may be conveniently drawn from the line according to the production rate. One aflatoxin assay should be run on each lot of 500 cases, 6 No. 10 cans or equivalent in other can sizes (20,625 pounds peanut butter). This should be an approximate one pound composite drawn at six different equally spaced intervals.

Lot Sampling -- The number of aflatoxin determinations should be one for each 500 cases, 6 No. 10 cans or equivalent. These should be composite samples made from the samples drawn for grade determination.

C. Peanut Meal

(200,000 pounds maximum size lot PAC) The handler shall have the Federal or Federal-State Inspection Service draw two ten pound samples designated as M-1 and M-2 which shall be forwarded to the AMS laboratory. If Sample M-1 and M-2 will not accept the lot, the handler may cause Sample M-3 and M-4 of ten pounds each to be drawn. For PAC an 1100 gram sample shall be analyzed.

D. Brazil Nuts and Pistachio Nuts (Imported)

Refer to Fresh Fruit and Vegetable Inspection detailed sampling plan specified for Brazil and Pistachio Nuts (2).

E. Appeal Sampling Shelled Peanuts

Appeal sampling shall be done by the Federal or Federal-State Inspection Service in accordance with PAC regulations. The sample size shall be 144 pounds of shelled peanuts which when ground in a "Dickens Subsampling Mill" will yield a 3300 gram ±300 grams subsample. The sample shall be sent to an AMS Laboratory or a laboratory designated by the PAC as an approved laboratory and shall be accompanied by Form FV-187 (Notice of Sampling).

F. Fragmented Peanuts (Segregation 3 peanuts)

The handler shall have the Federal or Federal-State Inspection Service draw one 48 pound sample designated as Sample "F". Sample "F" is ground in a "Dickens Subsampling Mill". The amount from the subsample should be about 1100 grams.

G. In-Shell Peanuts

The handler may, at his option, request that the Federal-State Inspection Service sample 10% of the containers in a lot resulting in a 48 pound sample of shelled kernels designated as Sample 1. This sample shall be placed in a suitable container and positively lot identified. The sample is shelled prior to grinding. Sample number one is ground in a "Dickens Subsampling Mill." The amount from the subsample output should be about 1100 grams and this shall be designated as Subsample 1-AB. If Sample 2 or Sample 3 is requested, there shall be 48 pounds of shelled kernels for each sample.

H. Blanched Peanuts

Sampling of blanched peanuts shall be done by the Federal or Federal-State Inspection Service. For lots of 60,000 pounds or less, a minimum of 3 samples of 12 pounds each shall be drawn. For lots exceeding 60,000 pounds, a minimum of 5 samples of 12 pounds each shall be drawn. Each sample is ground separately and an aflatoxin analysis is run on each sample. Each lot shall be accompanied by Form FV-187 (Notice of Sampling).

I. Corn

Sampling of corn to be officially analyzed and certified will be done by Fruit and Vegetable Division inspectors or by licensed inspectors as defined in File Code 109-A-1, Regulations Governing Inspection and Certification of Processed Fruits and Vegetables and Related Products. A minimum of 10 - one pound subsamples shall be drawn randomly and

composited into one sample for analysis. The sample shall be passed through a Dickens Mill and the ground sample shall be subsequently ground finer by grinding in a hammer mill. After grinding, the sample shall be thoroughly mixed and a representative 50 gram portion taken for analysis.

J. Other

The following shall serve as a guide for sampling products not covered in the categories above. These sampling rates are the minimum needed to insure a representative sample. Additional sample units should be drawn if necessary.

PRODUCT	LOT SIZE	NUMBER OF SAMPLE UNITS	UNIT SIZE	TOTAL SAMPLE SIZE
Tree Nuts 1/	Any size	10	1 lb	10 lbs
Brazil Nuts (in-shell)	Less than 200 bags	20	1 lb	20 lbs
	201 - 800 bags	40	1 lb	40 lbs
	801 - 2000 bags	60	1 lb	60 lbs
Pistachio Nuts (in-shell)	up to 75,000 lbs	minimum of 20% of containers	--	50 lbs
	75,001 - 150,000 lbs	minimum of 20% of containers	--	100 lbs
Pistachio Nuts (shelled)	up to 75,000 lbs	minimum of 20% of containers	--	25 lbs
	75,001 - 150,000 lbs	minimum of 20% of containers	--	50 lbs
Oil seed Meals	Any size	20	1 lb	20 lbs
Edible seeds-melon, pumpkin, etc.	Any size	50	1 lb	50 lbs
Small grains-wheat, barley	Any size	10	1 lb	10 lbs
Dried fruit	Any size	50	1 lb	50 lbs

1/ except in-shell Brazil Nuts and Pistachio Nuts

III ANALYTICAL PROCEDURE

The method used to analyze raw shelled peanuts, peanut meal, peanut butter and roasted peanuts for aflatoxin is the BF Method (3). The method has been modified to incorporate the water slurry method of extraction (4). The method is described below. It is intended for use by individuals who are familiar with analytical chemical techniques and equipment.

A. Water Slurry (Modification of BF Method)

1. Extraction and Cleanup

   a. Thoroughly mix and weigh an 1100 gram sample ± 100 grams, of peanuts or peanut meal and record the weight to the nearest gram. Place the sample in a 1 gallon stainless steel blender container and add 1600 ml water. Add the meal first, then the water. Blend for 3 minutes at medium speed to form a slurry. Within 2 minutes after blending, weigh 196 grams of the slurry into a 1 or 2 quart blender container.

   Add 283 mls of a 77 percent methanol solution and 160 ml of hexane. The 77 percent methanol solution is made by mixing 770 ml of methanol with 230 ml of 5 percent salt solution. The extract solution will contain 55 percent methanol. Blend for 30 seconds at high speed in an explosion proof blender.

   b. Transfer a portion to either a 500 ml or 1000 ml centrifuge bottle and centrifuge 10 - 20 minutes at 2000 rpm. If mixture has not separated into two distinct layers, centrifuge an additional 5 to 10 minutes. With a 100 ml transfer pipet, extract two 75 to 100 ml aliquots of the methanol-water layer. Filter each portion through a rapid filter paper, collecting the filtrate in an erlenmeyer flask. With a transfer pipet, accurately pipet two 50 ml aliquots of the filtrate into each of two 125 ml separatory funnels. (250 ml separatory funnels may be used if desired.) Add 50 ml chloroform and shake 30-60 seconds. Let the layers separate and drain the bottom chloroform layer into a 250 ml or larger stainless steel beaker. Discard the solvent remaining in the separatory funnel. Place the beaker on a water bath and evaporate to near dryness. Each beaker containing one of the two 50 ml aliquots from the same sample must be evaporated separately from the other beaker. If several samples are to be analyzed simultaneously, one test from each sample may be evaporated at the same time on the water bath and then followed by the duplicate samples. Do not leave the beaker on the water bath after evaporation.

   Transfer the residue in the beaker carefully to a 25 ml glass stoppered erlenmeyer flask or a vial with three small chloroform rinses, using a wash bottle. Place the flasks or vials on a steam bath and evaporate to just dryness. The duplicate tests from each sample must be evaporated separately.

2. Thin Layer Chromatography (TLC)

a. Preparation of Plates

Wash thoroughly and dry five 20x20 cm glass plates. Weigh the amount of silica gel as recommended by the manufacturer for the preparation of five plates into a 250 to 500 ml glass stoppered erlenmeyer flask. Add recommended amount of water, shake vigorously for one minute and pour into applicator. Immediately coat the five 20x20 cm glass plates with 0.25 mm thickness of silica gel suspension and let plates rest undisturbed until gelled (about 10 min.). If the slurry is not of the proper consistency to spread evenly, wash and dry the plates, prepare another slurry by adjusting the amount of water, and recoat the plates. Dry the coated plates 2 hours at 110°C and store in a desiccating cabinet until needed. The plates may need to be re-dried prior to use to reactivate the silica gel if the plates have been stored for several days. Commercially prepared pre-coated plates may be used in lieu of preparing plates.

b. Spotting Samples on TLC Plates

To prepare a plate for chromatography, scribe a line 16 cm from the bottom to act as a stop for solvent and 0.5 cm from each side to prevent edge effects. Some analysts scribe a line in the center of the plate as a solvent stop and re-use the plate by rotating it 180 degrees. This is permissable providing good separation is obtained for the four aflatoxins. Add 300 ul benzene-acetonitrile (98+2) to each sample flask or vial; stopper, and swirl to dissolve sample extract. Starting from the left side, spot from the first sample a 5.0, 2.0 and 6.5 ul spot in the order listed.

On top of the 5.0 ul spot, also spot 3 to 5 ul of the reference standard as an internal standard. For the remaining samples to be placed on one plate, spot only the 2.0 and 6.5 ul spots. Once again the duplicate tests from each sample must be spotted and developed on separate plates. The spots should all be approximately the same diameter (0.5 cm or less) and approximately 1 cm apart. In the center of the plate, after working from left to right, spot 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 uls of the reference standard. Continue spotting samples to the right of the standard spot. For the last sample, spot a 2.0, 6.5 and 5.0 ul spot. On top of the 5.0 ul spot also spot 3 to 5 ul of the reference standard as an internal standard.

At the discretion of the analyst, a 1.0 ul spot may be used in place of the 2 ul spot and a 5.0 ul spot may be used in place of the 6.5 ul spot.

c. Plate Development

Pour approximately 35 ml of the developing solution (see note 10, Section III.F.) into the trough which is placed against the back side of the tank. Place the plate in the trough with the upper edge leaning against the back of the tank. Close the cover, seal and develop for approximately 25 minutes. The developing time will vary depending upon laboratory conditions. An equilibrated tank will shorten the developing time over that required by an unequilibrated tank. It is necessary to obtain complete separation of the aflatoxins. Over-development will cause the aflatoxins to "bloom", which will increase the difficulty in estimating the intensity of the spots. Often, depending upon the nature of the silica gel, some developing solutions are preferred over others and one should refer to Chapter 26 of the AOAC for these other systems. After the plate has been developed, remove from the tank and allow to dry prior to reading the plate. The plate must be completely dry before exposing it to UV light.

d. Interpretation of the Chromatogram

Examine the plate under a long wave UV viewing chamber. Four clearly identifiable spots should be visible in the resolution reference standard.

Examine the pattern from the sample spots containing internal standard for aflatoxin spots. Rf values of aflatoxins used as internal standards should be the same as, or only slightly different from, those of respective aflatoxin standard spots.

Compare sample pattern with pattern containing internal aflatoxin standard. Fluorescent spots in sample thought to be aflatoxins must have Rf values identical to and color similar to aflatoxin standard spots when unknown spot and internal standard spot are superimposed. Compare fluorescent intensities of B1 spots of the sample with those of the standard spots and determine which standard spot matches the sample.

Interpolate if the intensity of the sample spot is between those of two (2) standard spots. If spots of the smallest portion of the sample are too intense to match the standards, evaporate sample to dryness and redilute using a larger portion of benzene-acetonitrile solution. Compare B2, G1 and G2 in a similar manner.

3. Calculations

Calculate the concentration of aflatoxin B1 in parts per billion from the following formula:

parts per billion = (SxYxV) / (XxW)

Where S=ul aflatoxin B1 standard equal to unknown; Y=concentration of B1 standard (ug/ml); V=ul of final dilution of sample extract; X=ul of sample extract spotted giving fluorescent intensity equal to S(B1 standard); W=grams of sample contained in aliquot transferred to separatory funnel.

If it is possible to read both sample spots against the standard, they should be calculated and the average taken as the final reading. The same procedure should be followed in calculating the concentrations of the B2, G1 and G2 spots. The total aflatoxins will be the total of the individual spots expressed to the nearest whole number.

Calculate W as in the following example, using a sample size of 1100 grams and a 50 ml aliquot to the separatory funnel.

```
 1600g  water (assuming a density of 1g/ml for water)
+1100g  peanuts
 2700g  total

  196g x 1100g = 79.85g peanuts
 2700g

 196.00g  slurry
-79.85g   peanuts
 116.15g  water

 283.00 ml of 77% methanol solution
+116.15 ml water
 399.15 ml total

  79.85g x 50 ml = 10.0g peanuts
 399.15 ml
 This is W (grams of sample contained in aliquot)
```

Since the sample size may vary between 1000g and 1200g, the amount of water added to the sample must be varied accordingly, to result in a slurry that contains a constant ratio of peanuts to water. Obtain the correct amount of water to add from the chart in attachment II. It is important that W be equal to 10 grams.

Attachment III may be used as an aid in calculating aflatoxin when using The Water Slurry Modification of the BF Method.

B. CB METHOD

An alternative method is Method I [(CB Method) as described in the AOAC, Chapter 26 revised March 1980]. The CB Method must be used where reasonable doubt exists as to the effectiveness of the BF method in extracting aflatoxin from the sample. Examples of doubtful results are those in which excessive background material makes quantitation impossible, or there exists in the 25 ml erlenmeyer flask, a residue which does not dissolve in the spotting solution of benzene-acetonitrile (98+2). The CB Method must be used for corn samples and for samples of peanut butter which exceed 6 ppb total aflatoxin when analyzed by the BF Method.

1. Extraction

Weigh 50 grams of prepared sample into a 500 ml erlenmeyer flask. Add 25 grams diatomaceous earth (Hyflo Super-Cel or equivalent), 25 ml water, and 250 ml chloroform. Stopper the flask and secure the stopper with tape. Shake 30 minutes on a wrist action shaker and filter through fluted filter paper. If filtration is slow, transfer to a buchner funnel coated with about a 5 mm layer of diatomaceous earth and use a light vacuum. Use this procedure only for slow filtering samples since vacuum speeds the evaporation of chloroform, resulting in concentration of the extract. Collect the first 50 ml portion of chloroform filtrate for the cleanup step.

2. Column Chromatography

Place a ball of glass wool loosely in the bottom of a 22x300 mm chromatographic tube and add about 5 grams anhydrous sodium sulfate to give a base for the silica gel. Then add 10 grams E. Merck Silica Gel 60 (Brinkman Instruments, Inc. Catalog number 7734 or equivalent). Wash the sides of the tube with about 20 ml of chloroform and stir to disperse silica gel. Drain some of the chloroform to aid settling, leaving about a 5 cm layer of chloroform above the silica gel. Carefully add a 1 to 2 cm layer of anhydrous sodium sulfate. Drain the chloroform to leave the chloroform about 1 cm above the top of the sodium sulfate. Add the 50 ml of sample from the extraction step to the column and elute at maximum flow rate with 150 ml of hexane, followed by 150 ml of anhydrous ethyl ether and discard. (see note 7)

When the ether is about 2 cm above the top of the sodium sulfate, add 150 ml of methanol-chloroform (3+97) to elute the aflatoxins. At this time, place a stainless steel beaker beneath the column to collect this fraction until the flow stops. After collecting this fraction, evaporate to nearly dryness on a steam bath. Transfer carefully to a 25 ml glass stoppered erlenmeyer flask or vial with three small rinses of chloroform. Place flasks or vials on a steam bath and evaporate to just dryness. Follow the procedure for the BF method beginning with the section "THIN LAYER CHROMATOGRAPHY." Duplicate samples are unnecessary as raw peanuts are not analyzed by this method for the PAC.

C. Confirmation of aflatoxins G1 and/or G2

1. This is applicable only when a commodity such as peanut butter contains interferences that tend to migrate in the aflatoxin G area. If G1 plus G2 is equal to or greater than 20 percent of the total aflatoxin, confirm the amount of G1 and G2 by using benzene-ethanol-water (46+35+19) as the solvent system. Shake the benzene-ethanol-water in a separatory funnel and allow it to set overnight. Carefully separate the upper and lower phases. The upper phase is used in the trough and the lower phase in the bottom of the tank. Respot the sample and standard on a silica gel plate as before in III.A.2.b.

2. Put 40 ml of upper phase in the trough and 40 ml of lower phase in the bottom of the developing tank. Place the trough against the back side of the tank so as to allow maximum exposure of plate surface to tank volume. Seal and let solvent solution rise approximately 25 minutes, or according to laboratory conditions for proper separation of aflatoxins. B1, B2, G1, and G2 will be in the same order as before, but much of the extraneous fluorescence will have different Rf's relative to those aflatoxins in the two solvent systems. The G1 and G2 aflatoxins of the sample should have the same Rf's as those of the respective standards. Make a quantitative estimate for G1 and G2 as in III.A.2.d (Interpretation of the Chromatogram).

D. Confirmation of Aflatoxin by Derivative Formation

Occasionally, blue or green fluorescent spots appear that are questionable as to identity. To confirm the identity of the spots, the following procedure is used.

Divide the silica gel on a plate vertically by scoring a line down the center. On the left hand side of the plate spot two 3 to 5 ul aliquots of sample extract. On one of the sample spots, add a 3 to 5 ul spot of standard. To the right of the two spots, place a 3 to 5 ul spot of standard only.

To each of the spots (sample only, sample plus standard and standard only), add 1 ul of trifluoroacetic acid (TFA). After spotting with TFA, immediately rinse syringe thoroughly with soapy water and then with benzene-acetonitrile (98+2). Place the plate in a dark area and let the TFA react for 5 minutes. Dry the plate by placing in a warm oven (100° to 120° C) for 10 minutes or by blowing warm air over the plate for 10 minutes.

On the right hand side of the plate, spot sample and standard identical to the way it was done on the left hand side, however do not place TFA on the spots. Develop the plate in the normal manner and after drying, examine under UV light. The right hand side of the plate should look as it normally does. On the left hand side of the plate, the aflatoxin derivatives should appear at Rf's about one-fourth of the aflatoxin Rf's on the right hand side. Sample Rf's that match those of the standard confirm the presence of aflatoxin. However, if the sample has migrated to the same position as the sample on the right, the sample does not contain aflatoxin.

Spraying a plate with acid is another way to aid in determining the absence of aflatoxin. If a plate is suspected to contain spots that may not be aflatoxin, spray the plate with sulfuric acid-water (1+3). The acid changes the aflatoxin fluorescence from blue or green to yellow. Spots which do not turn yellow are not aflatoxin. The test only confirms the absence of aflatoxin. Spots which turn yellow may or may not be aflatoxin.

E. Standards

Aflatoxin standards are obtained from the Washington laboratory. They are dissolved in benzene-acetonitrile (98+2) in the following concentrations:

B1 - 1.0 ug/ml

B2 - 0.3 ug/ml

G1 - 1.0 ug/ml

G2 - 0.3 ug/ml

The standards require no further dilution and have been standardized by the spectrophotometric method described in Chapter 26 of the AOAC. Each vial contains approximately 8 ml of standard solution. Two or three ml of the standard may be kept in a glass vial with foil lined screw cap lid. Store the vial under refrigeration and use it to periodically check the working standard. The working standard is the 5 or 6 ml remaining in the vial. Transfer this to a volumetric flask with a glass stopper which is placed in a jar, protected from light, containing cotton saturated with benzene-acetonitrile solution. A red colored volumetric flask offers additional protection from light. Alternatively, the standard may be kept in an amber colored reaction vial fitted with a valve. The standard solution should be kept refrigerated when not being used, however, bring it to room temperature before opening.

F. Technical Notes

1. In the final step, the sample extract is diluted with 300 ul of benzene-acetonitrile (98+2) in a 25 ml glass stoppered flask or a vial. For dilution, use a 1 ml pipet graduated in 1/10 ml or an automatic pipet which delivers 300 ul. Pipettes should be calibrated prior to using.

2. TLC development -- The plate should be left in the sealed tank long enough to permit the B1 spot to migrate approximately 6 to 9 cm from the origin. It is not necessary for the solvent front to reach the 16 cm mark. The plate should be developed long enough to effectively separate the spots, however overdevelopment causes spreading or diffusing of the spots. Temperature and humidity conditions may change the development time and also may necessitate the change to a different developing solvent. Therefore, analysts must experiment to find the proper length of time for developing and the proper solvent to use. Benzene should be avoided as a developing solvent because it is a known carcinogen. Developing of the plates should be done under a fume hood regardless of which developing solvent is used.

3. Calculations -- Water Slurry Method -- If the intensity of the 6.5 ul sample spot is equal to the intensity of the 1.0 ul standard spot, and the 50 ml aliquot contained 10.0 grams of original sample, the calculation is as follows for B1 or G1.

$$\frac{1.0 \text{ ug/ml} \times 1.0 \text{ ul} \times 300 \text{ ul}}{6.5 \text{ ul} \times 10 \text{ g}} = 4.6 \text{ ppb}$$

A similar calculation for B2 or G2 would be as follows:

$$\frac{0.3 \text{ ug/ml} \times 1.0 \text{ ul} \times 300 \text{ ul}}{6.5 \text{ ul} \times 10 \text{ g}} = 1.4 \text{ ppb}$$

4. Calculations -- CB Method -- Similar to above, except that the equivalent weight of original sample is 10 grams which is further corrected to 9 grams for peanuts. This is because the 50 ml aliquot for column chromatography contains approximately 5 ml of fat.

5. If replating is necessary, and the sample flasks have been kept tightly stoppered, it is unnecessary to evaporate and redilute the sample. However, if evaporation of any solvent is suspected, the sample extract must be evaporated on a water bath and rediluted to a suitable volume. Also, if the sample must be made more dilute because the smallest sample spot is brighter than the largest standard spot, the sample extract must be evaporated and diluted to the concentration desired. In the calculation of the replated results, a correction for the loss in volume of the sample extract is necessary. For example, in the original spotting 2.0 uls and 6.5 uls for a total of 8.5 uls were used. The original volume was 300 ul. Therefore, there was a balance remaining of 291.5 ul. This results in the following factor:

$$\frac{291.5}{300} = 0.97$$

The final answer found will have to be divided by this factor to compensate for the amount of sample used in the original plating.

If the replated value found is 25 ppb, then the corrected value will be $\frac{25}{0.97} = 25.8$ ppb.

6. Sometimes excessive background fluorescence can mask the aflatoxin spots, making them difficult to quantitate. Also, at times the residue may not be entirely soluble in the spotting solvent and no additional sample remains with which to run the CB Method. In either case, the following clean-up procedure may be used.

Prepare a butt tube (Curtin-Matheson Scientific, Inc., Catalog No. 087-411 or equivalent) as follows:

a. Tamp a wad of glass wool into the butt tube.

b. Add a dry layer of anhydrous sodium sulfate about 1 cm in depth.

c. Add about 6 grams silica gel 60 (Brinkman Instruments, Inc. Catalog No. 7734 or equivalent).

d. Add another dry layer of anhydrous sodium sulfate about 1 cm in depth.

e. Wet the column with chloroform using a wash bottle and tap the top layer to level.

f. Add a wad of glass wool at the top of the butt tube.

After carefully evaporating the spotting solvent from the sample, add about 5 ml of chloroform-acetone (9+1) to the flask containing the sample. Place a stainless steel beaker under the butt tube. Transfer the sample solution to the butt tube, keeping the top layer as level as possible. Rinse the flask with about 5 ml of chloroform-acetone (9+1) and add to the tube. Add 125 ml of chloroform-acetone (9+1) to the tube. Evaporate the filtrate collected in the beaker and transfer to the sample flask for evaporation, dilution and spotting. If possible, use the same sample flask as any residue not rinsed out will still remain and prevent loss of aflatoxin.

7. Use only ethyl ether containing a trace (.01%) of ethanol. Some ether contains 2% ethanol added as a preservative, however, it is not satisfactory as it will strip the aflatoxin from the column during the cleanup step.

8. Keep spotting syringes sharp at all times. When the needle becomes dull replace it immediately, as a dull needle will pick up silica gel containing part of the sample from the surface of the plate causing erroneous results. If the needle picks up a noticable piece of silica gel while spoting the sample, place another spot down beside it to insure that you have an accurate spot.

9. Gels

The AOAC Volume 52, No. 2 (1969), page 252, describes a satisfactorily developed plate as one on which "Four aflatoxins be separated from each other in clearly defined spots and separated from non-aflatoxin fluorescent materials in the extract of the commodity being examined."

Any silica gel that meets this criteria may be used. Listed below are some of the more effective gels.

a. Brinkmann G-HR

May be used on raw peanuts, roasted peanuts, peanut butter and peanut meal.

b. Absorbosil 1 and/or 5

May be used on raw peanuts, peanut butter, brazil nuts, pistachio nuts and peanut meal.

c. Silica 7G

May be used, but background material may make it hard to evaluate aflatoxin spots.

Mixing should be done vigorously for one minute. If the mixture is too thin, mixing for an additional 15 or 30 seconds may correct this.

The gel should be applied to a minimum thickness on the plate (between 25 and 35 mm).

Development time varies with the type of gel, thickness of the gel, developer used, and room temperature. The analyst will have to determine the optimum condition for his laboratory. A development time of over 40-50 minutes is excessive, 20-30 minutes is ideal.

Each batch of gel, and sometimes individual containers of gel within a batch, may vary. If a gel does not produce a good plate, activating the gel at 80°-100° C. for an hour or so before use may restore the gel.

The gel systems on the dried plates are extremely hygroscopic and even a little moisture can reduce the effectiveness of the plate. Preheating each plate for 15-20 minutes before spotting is a good practice.

There are a number of pre-coated plates that are commercially available that may be used instead of preparing plates.

10. Developing Solutions

Developing systems must be tailored to each laboratory since temperature, humidity, and many other variable conditions may cause plates to develop differently in various locations. Developing solutions consist of one or more polar or non-polar solvents. All solvents exhibit some polarity, however some solvents exhibit very little polarity and are thus considered non-polar. The following is a list of some polar and non-polar solvents:

Polar Solvents	Non-polar Solvents
Water	Chloroform
Methanol	Ethyl Acetate
Acetic Acid	2-Propanol
Acetonitrile	Methylene Chloride
Acetone	Toluene
Ethanol	Hexane

From the diagram, it is seen that water is the most polar solvent on the list and hexane is the most non-polar. This information can be used when selecting a developing system, or adjusting the concentrations of solvents in the system being used, because the movement of aflatoxins on the plate is suppressed by non-polar solvents and enhanced by polar solvents. For example, if you are using a developer consisting of 90% chloroform and 10% acetone and find that the spots move too far, reduce the amount of acetone. If the spots do not move enough, increase the amount of acetone. Benzene should never be used as a developer except for the confirmation of aflatoxins G1 and G2 (see III.C.), because of the health hazards associated with benzene.

The following is a list of developing solutions which may be used:

1. 90% Chloroform + 10% Acetone
2. 90% Chloroform + (5-15%) Methanol
3. 85% Chloroform + 5% Acetic Acid + (10-15%) Ethyl Acetate
4. 50% Toluene + 10% Acetic Acid + 40% Ethyl Acetate
5. 80% Toluene + 10% Acetic Acid + 10% Methanol
6. 80% Toluene + 22% Acetone + 16% Acetic Acid

IV CERTIFICATION

PEANUTS, SHELLED

Because of error inherent in sampling and testing and the difficulty of duplicating test results on samples drawn from the same lot, there is some question regarding the reporting of exact values on inspection certificates. The PAC and USDA have agreed that some administrative discretion should be applied in reporting values of 25 ppb or less. This section outlines certification procedures and how to report aflatoxin values. If the applicant specifically requests the actual test results in ppb, it may be reported by telephone.

A. Domestic Shipments

1. On lots where USDA makes the initial inspection on Sample 1 AB:

   a. If the average value for Sample 1A and 1B is 16 ppb or less, issue a certificate and show -- AFLATOXIN - "NEGATIVE".

   b. If the average value for Sample 1A and 1B is greater than 75 ppb, issue a certificate showing the average ppb.

   c. If the average value for Sample 1A and 1B is 17 thru 75 ppb, request Sample No. 2 AB.

d. If the average value for Sample 1A, 1B, 2A and 2B, is 22 ppb or less, issue a certificate and show -- AFLATOXIN - "NEGATIVE".

e. If the average value for Sample 1A, 1B, 2A and 2B is 38 ppb or more, issue a certificate showing the average ppb.

f. If the average value for Sample 1A, 1B, 2A and 2B is 23 thru 37 ppb, request Sample No. 3 AB.

g. If the average value for Sample 1A, 1B, 2A, 2B, 3A and 3B is 25 ppb or less, issue a certificate showing -- AFLATOXIN - "NEGATIVE".

h. If the average value for Sample 1A, 1B, 2A, 2B, 3A and 3B is more than 25 ppb, issue a certificate showing average ppb.

The following chart may be used as an aid in certification:

Subsample	Certify as Negative	Request next Subsample	Positive - Certify Showing Results
1AB	0 - 16	17 - 75	76 and higher
1AB + 2AB	0 - 22	23 - 37	38 and higher
1AB + 2AB + 3AB	0 - 25	--------	26 and higher

results are shown in ppb.

2. Sample 1 CD

When this sample is sent from the number 1 sample to a manufacturer which has an approved PAC "36 Hour Lab", the manufacturer can accept the lot based on his findings and we would not run any samples. If the manufacturer elects not to accept the lot, he will notify the Federal-State Inspection Service and the handler to send Sample 1 AB to the USDA Laboratory. We will handle the same as domestic shipments. The results of the 1 CD sample will not be averaged with our findings. The manufacturer is never to be allowed the CD sample on sample number 2 or 3.

3. FV 187 (Issued by Fresh Fruit and Vegetable Branch)

A copy of each FV-187 on each lot of shelled peanuts (Farmers Stock, Oilstock, Meal and Peanut Butter excluded) should be sent to:

Peanut Administrative Committee
P.O. Box 18856
Lenox Square Station
Atlanta, Georgia 30326

4. PAC

The PAC needs to have the exact values found on all samples analyzed for this program. Every lot of officially sampled peanuts has an FV-187 issued for it. The following should be added to the FV-187 by the analyst, using a rubber stamp to stamp the FV-187 and writing in the applicable aflatoxin levels.

This rubber stamp will also be used to stamp the copy of the certificate going to PAC. The aflatoxin levels will be written in as is done on the FV-187.

Aflatoxin Analysis AMS Certificate No.			Aflatoxin Analysis AMS Certificate No.			Aflatoxin Analysis AMS Certificate No.		
1A	10	PPB	1A	18	PPB	1A	28	PPB
1B	9	PPB	1B	20	PPB	1B	30	PPB
2A		PPB	2A	18	PPB	2A	30	PPB
2B		PPB	2B	22	PPB	2B	32	PPB
3A		PPB	3A		PPB	3A	28	PPB
3B		PPB	3B		PPB	3B	32	PPB
Avg.	10	PPB	Avg.	20	PPB	Avg.	30	PPB
(Example of 1A & 1B analyses)			(Example of 1A, 1B, 2A & 2B analyses)			(Example of 1A, 1B, 2A, 2B, 3A & 3B analyses)		

Draw a line across lines not used -- see example above.

If a FV-187 is furnished, the Certificate (FV-146) sent to PAC under this program should contain the following information.

a. Poundage (i.e.) 40,200 pounds

b. House number, lot number, and crop year.

If no FV 187 accompanies the sample, contact your supervisor who will in turn notify the Washington office through the regional director.

B. Foreign Shipments

1. Canadian: All U.S. No. 1 or better will be certified exactly as described for domestic shipments.

2. Other Foreign: (Export CCC Program Announcement) Sample 1 AB is analyzed upon receipt. If the average of 1A + 1B is 16 ppb or less, issue a certificate showing AFLATOXIN - "NEGATIVE". If the average is greater than 75 ppb. issue a certificate showing the average ppb. If the average is more than 16 ppb, but not more than 75 ppb, the applicant should be notified. If the applicant chooses to cease testing, a certificate is issued showing the average ppb aflatoxin and stating "Fails to meet requirements of Announcement PV-V-FS-1 dated November 20, 1976," in the grade statement. If the applicant elects to have subsample 2 AB analyzed and the average of 1A+1B+2A+2B is 22 ppb or less, issue a certificate showing Aflatoxin "NEGATIVE."

   If the average is 38 ppb or greater, issue a certificate showing the average ppb. If the average is greater than 22 ppb, but less than 38 ppb, the applicant should be notified. If the applicant chooses to cease testing, issue a certificate showing the average level of aflatoxin in ppb and stating "Fails to meet requirements of Announcement PV-V-FS-1" dated November 20, 1976. If he elects to analyze subsample 3AB and the average of 1A+1B+2A+2B+3A+3B is 25 ppb or less, issue a certificate showing AFLATOXIN - "NEGATIVE." If the average is greater than 25 ppb, issue a certificate showing the average ppb. (The applicant pays for all analyses).

C. Peanut Butter

All samples tested for aflatoxin must meet current FDA acceptance tolerances. In no instance may one sample exceed the tolerance and the lot be accepted because the average meets the limit.

If a lot fails the tolerance, notify your field office and Washington. Send samples to Washington for verification testing prior to notifying the applicant that the lot failed.

1. USDA purchases will be certified as follows:

   MEETS -- Call the inspector at the plant if the results are within the current FDA guideline and send a copy of the certificate for his records. If all quality requirements are met, the inspector at the plant will issue a certificate as follows:

   > "Product meets all quality requirements of Announcement FV---." He will make no mention of aflatoxin on the certificate.

   FAILS -- Call OIC at once and hold the balance of the sample for Washington. Release no information until instructed by the OIC or Washington.

After being notified by the OIC or Washington of a failing lot of Peanut Butter:

The inspector will certify "Product fails quality requirements of Announcement FV--- account presence of aflatoxin." He will show results in the body of the certificate (i.e. Aflatoxin 52 ppb.) Unofficial samples may be analyzed for the applicant and a suitable report prepared.

If the applicant wants aflatoxin analysis only (no grade) show the aflatoxin value in the body of the certificate (i.e. Aflatoxin 17 ppb) and under Remarks: "Inspection restricted to aflatoxin analyses only." If the grade is requested also, show actual ppb found in the body of the certificate. If the aflatoxin is over the current FDA acceptable value, the product shall be "Grade Not Certified (GNC) - account aflatoxin exceeds FDA administrative guideline." Follow procedure in file code 172-A-1 for GNC lots.

D. Peanut Meal

Domestic

Sample M1 shall be analyzed and if the results are acceptable to PAC (16 ppb or less), a negative certificate shall be issued. If the result is considered excessive by the Committee (17 ppb or more), the handler will be contacted so he may exercise his option to cease testing. If the handler elects to cease testing, a certificate shall be issued showing "Restricted." If the handler elects to have Sample M2 analyzed, the results of M1 + M2 shall be averaged. If 25 ppb or less, a negative certificate shall be issued. If greater than 25 ppb, the handler shall again be contacted. If the handler elects to stop, a certificate shall be issued stating "Restricted." If he elects to continue, the Federal State Inspection Service, at the handler's request, may again draw, in the appropriate manner, two 10-pound samples designated as M3 and M4.

Sample M3 and M4 shall be analyzed at the same time and if the average of M1 + M2 + M3 + M4 is acceptable by the Committee (25 ppb or less), a negative certificate shall be issued. If the average of M1 + M2 + M3 + M4 is considered excessive (26 ppb or more), a certificate showing the average ppb shall be issued. All analyses shall be charged to the account of the applicant.

E. Brazil and Pistachio Nuts

If the results of the shell and kernel analysis are greater than 20 ppb, the laboratory shall notify the handler of the results and a second sample shall be analyzed. This analysis shall be on kernels only and if the results are greater than 20 ppb, a certificate shall be issued showing the actual ppb. If less than 20 ppb, a negative certificate shall be issued. No lot shall be resampled when a chemical assay has been made on kernels only.

F. Fragmented Peanuts (Segregation 3 peanuts)

The 1100 gram sample will be prepared the same as for 1 AB (2 aliquots) and the average numerical results for F1 and F2 will be shown on the certificate. Never use the term "Negative", when certifying fragmented peanuts.

G. Other Categories

All Oil Stock

The acceptance and rejection plan is the same as for shelled peanuts, except that after running Sample 1A + 1B and the lot is not acceptable, the applicant has the option of stopping. If the applicant elects to stop, a restricted certificate shall be issued with the average ppb shown. If he elects to continue the assay, he may again stop after Samples 1A + 1B + 2A + 2B have been analyzed. If the lot is not acceptable, a restricted certificate shall be issued with the average ppb shown. If he elects to continue, 3AB shall be analyzed and the average of 1A + 1B + 2A + 2B + 3A + 3B shall be used for the basis of accepting or rejecting the lot.

H. In-Shell Peanuts

When the handler requests aflatoxin testing of In-Shell peanuts, handle the same as for Oil Stock.

I. Appeal Inspection - Shelled Peanuts

The sample shall be divided on an approved sample splitter into three samples of approximately 1,100 grams each and each subsample shall have two tests run. The results will be listed on the certificate showing the actual ppb for each sample.

Acceptance and rejection will be done by the PAC. All costs shall be paid by the handler and the certificate will be made out to the applicant. A copy of the certificate and a copy of form FV-187 shall be sent to the PAC.

J. Blanched Peanuts

Show the results for each aflatoxin test on the certificate. There will be 3 test results for lots of 60,000 pounds or less and 5 test results for lots exceeding 60,000 pounds. PAC will accept or reject the lot based on the results shown.

K. Financially Interested Parties

At the applicant's request, we may provide daily, the numerical values on all samples we have completed. The information can be given by phone, telegram or other method specified, at the applicant's expense.

If desired, the remarks section of the certificate may indicate that the handler, applicant or receiver was notified, and the date and time this was done.

EXAMPLE: Applicant notified of results at 1430 hours on April 1, 1975, by telephone.

It is preferable to show this information on the ledger, rather than the certificate.

L. Recertification

Follow instructions in file code 165-A-1.

M. Unofficial Samples

If the sample is unofficially submitted, certify as: "CERTIFICATE RESTRICTED: Samples submitted by applicant and do not officially represent any lot" should appear under the grade statement. Source of samples should be shown as "Submitted by Applicant."

N. Peanuts for USDA Contracts

When an aflatoxin certificate is over 30 days old on a lot of peanuts, a new aflatoxin analysis must be run. The procedure shall be the same as in IV. I. Appeal Inspection - Shelled Peanuts. The six results shall be shown on the certificate and a copy will be sent to the processed products inspector at the peanut butter or peanut granule plant.

O. Other

Show the actual value found in the body of the certificate and restrict the certificate to aflatoxin analysis only.

P. Billing

See attachment 1 for the charges to be assesed for various types of analyses, and the procedure for billing the PAC or the applicant.

V SAFETY

A. General

Aflatoxin has been shown to be an extremely potent toxic material to many animals. Neither the effects of aflatoxin on man nor all the possible routes of entry are presently known, but this material is considered to be the most potent carcinogen that we know of today. Manipulations should be carried out under fume hoods whenever possible, and particular precautions should be taken when the toxin is in the dry form because of its electrostatic nature and resulting tendency to be dispersed in working areas.

There are also hazards present in the laboratory from the use of organic solvents. For this reason, safety should be the primary consideration when performing analysis for aflatoxin. If equipment is malfunctioning or a safety hazard is noted, all work must cease until the problem is corrected and the laboratory has been determined to be safe to work in.

B. Aflatoxin

The following are particular precautions to be taken for aflatoxin:

1. Spillage

The area affected should be completely covered with 5% sodium hypochlorite (undiluted household bleach). If the volume of any spilled solution is known, the volume of bleach applied should be at least one-tenth the volume of spilled solution. Contact time should be at least 30 seconds.

2. Glassware

All glassware should be decontaminated. If chromic acid cleaning solution is used, no other treatment is necessary. Since aflatoxin is destroyed by alkali and strong acids, cleaning procedures involving these types of reagents are sufficient for decontamination. TLC plate coatings should be thoroughly wet with bleach before they are washed off the plate. Sample flasks should be taken to dryness under the fume hood and wet with bleach. For general dishwashing, a solution of bleach diluted 10 fold with water should be used and the entire container should be wet with the solution for at least 30 seconds. After washing glassware, thoroughly rinse to remove all traces of bleach, as it could destroy future aflatoxin samples during analysis. Sample flasks should be oven dried to drive off the bleach. Oven drying of TLC plates after coating with silica gel is sufficient to remove bleach from them.

3. Work Area Surfaces

For dust and powder from toxic commodities such as peanut meal, the directions for spillage should be used. Centrifuges should occasionally be decontaminated with a 10 percent solution of bleach and water, however, they must be thoroughly rinsed to remove all traces of bleach. Always unplug centrifuges before cleaning. Fume hoods may be cleaned by mixing equal volumes of undiluted bleach and 6N hydrochloric acid and allowing the resultant chlorine gas generated to diffuse through the hood for a few minutes before turning on the exhaust fan. Keep the fume hood window down throughout this procedure and use extreme caution as chlorine fumes are toxic.

4. Personnel

Analysts handling aflatoxin should wear protective gloves routinely. Respirators should be worn when handling dry aflatoxin standards and when grinding samples of products that produce dust, such as corn. If aflatoxin comes in contact with the skin, wash the affected body parts immediately with undiluted bleach. In the event that the toxic material enters the mouth, a solution containing 1% sodium perborate and 1% sodium bicarbonate in water should be used as a gargle. See a physician immediately.

C. Organic Solvents

1. Benzene

One of the most dangerous chemicals used in aflatoxin testing is benzene. The fumes are highly toxic when inhaled. Also, skin and eye contact should be avoided with liquid benzene. At high concentrations, benzene causes a stimulatory effect, followed by depression, convulsions and death. If exposed to high concentrations of benzene, the victim should be given oxygen or artificial resuscitation. Flush the eyes and wash contaminated skin.

The long term effects of benzene are of particular concern to analysts in aflatoxin laboratories, since exposure to high concentrations should not occur if proper safety practices are observed. Unfortunately, benzene in very small doses can produce harmful long term effects to the bone marrow, with resultant anemia or leukemia. Some people are more sensitive than others to the effects of benzene. Consequently, medical monitoring is used as a guide to observe any changes that may be occurring in the body as a result of exposure to benzene.

Benzene is also extremely flammable. Underwriters' Laboratories fire hazard classification for benzene is 95 to 100 on a scale in which ether is the standard for 100.

For this reason, the use of benzene should be restricted to necessary operations. Do not use benzene as a developing solvent. Spot aflatoxin plates under a fume hood. After the analysis is complete and samples are no longer needed, evaporate the benzene-acetonitrile from the flasks on a water bath under the fume hood. Never pour benzene into the sink.

2. Chloroform

It is necessary to use in excess of 50 ml of chloroform for each analysis performed by the BF Method and approximately 400 ml of chloroform for each analysis using the CB Method.

Chloroform fumes are heavier than air and in high concentrations may cause unconsciousness and death. Therefore, it is imperative that a fume hood be used when working with chloroform. There also exists a fire hazard with chloroform. While it is not flammable itself, when chloroform is contacted by flame, it decomposes into phosgene which is extremely toxic when inhaled. Therefore, chloroform should be stored away from flammable solvents.

3. Other Solvents

There are several other organic solvents which have hazards associated with them including the following:

Hexane - A very flammable solvent, vapors are harmful to the respiratory system.

Methanol - A very flammable solvent, vapors are harmful to respiratory system. Ingestion of the liquid can cause blindness or death.

Diethyl Ether - An extremely flammable liquid. Upon exposure to sunlight, upon long standing or in the presence of oxygen, ether may form unstable peroxides which may explode spontaneously. When any of these conditions exist or for any other reason the ether is suspected of containing peroxides check for peroxides by shaking 9 ml of ether with 1 ml of saturated potassium iodide solution. If the purple color of the potassium iodide appears in the ether layer, peroxides are present and the ether is unsafe to use and must be disposed of. Breathing low concentrations of ether vapor causes unconsciousness.

Acetonitrile - A very flammable liquid, highly toxic, can be absorbed through the skin and respiratory tract. High concentrations cause rapid death. Also, the products of decomposition are highly toxic.

Acetone - A very flammable liquid, vapors are harmful to respiratory system.

Acetic Acid - Not combustable, but has hazardous vapors. The liquid causes burns when in contact with the skin.

Other solvents which may be used in developing solutions are toluene, ethyl alchol, ethyl acetate, and isopropyl alcohol. All of these solvents are hazardous with respect to harmful vapors and all are very flammable.

When working with any of the above solvents, use precautions to prevent fire. Keep only working quantities of solvents in the laboratory and store the remainder in a separate location. When transferring from one metallic container to another, make sure both containers are grounded. They must share a common ground to prevent sparks from igniting the solvent. Laboratories and storage areas should have prominent "No Smoking" signs displayed. Use only explosion proof blenders and centrifuges when running aflatoxin tests.

As mentioned, the vapors of most organic chemicals are dangerous and the liquid, in some cases, can be absorbed through the skin. The effects of these chemicals are often cumulative, causing destruction of bone marrow, disease of the liver, kidneys, and severe lung disorders. Some of the long term effects are probably still not known. Therefore, keep contact with these chemicals at a minimum. Work under a fume hood. Never pipet by mouth. Wear gloves when necessary.

D. Other Compounds

There are a few other substances in the laboratory that may be harmful. Dry silica gel powder has a fine particulate dust which can be harmful to the lungs. Diatomaceous earth used as a filter aid also has this danger. Glass wool can be harmful to the skin.

E. Laboratory Equipment and Layout

Particular caution should be used when operating the centrifuge. Always balance the load property and leave the lid closed and latched until the motor has come to a complete stop. A centrifuge is capable of generating hundreds of pounds of torque and should the heads come off, serious injury could result and considerable property damage could be done.

Observe good laboratory practices with respect to the blenders. Here again is a piece of equipment that generates considerable torque. Make sure the blender cup is situated securely on the spindle and that the lid is clamped tightly on the blender cup. When using a vacuum pump as a source of vacuum for pipetting, make sure the belts are covered with a protective device. When using the UV viewing box, care must be taken to avoid exposure to the eyes by UV radiation. All other equipment that is used in the laboratory should be operated according to the manufacturer's instructions.

1. NO Smoking!

2. NO Food in Lab

3. NO Drinking in Lab (Coffee, Water, Soft Drinks, etc.)

Refer to file code section 111 for additional safety precautions, especially 111-A-46.

VI REFERENCES

(1) "Aflatoxin Control Program for Peanuts" by J. W. Dickens, Journal of the American Oil Chemists' Society, Vol. 54 No. 3, March 1977, pp 225A-228A.

(2) Inspection Instructions for Sampling of In-Shell Brazil Nuts and Pistachio Nuts for Aflatoxin Analysis, November 1972, United States Department of Agriculture, Agricultural Marketing Service, Fruit and Vegetable Division, Fresh Products Branch.

(3) AOAC: Natural Poisons Chapter 26 from Official Methods of Analysis, 13th Edition, Association of Official Analytical Chemists, Washington, DC, 1980.

(4) "A Water Slurry Method of Extracting Aflatoxin from Peanuts" by T. B. Whitacker and J. W. Dickens, Journal of the American Oil Chemists Society, September 1980, pp 269 - 272.

JOE A. FLY
Branch Chief

Distribution:O
Agriculture:Washington

BILLING

A. Commercial Billing

A charge of one hour per analysis will be assessed the applicant for all official and unofficial samples analyzed by the BF Method except, there will be a charge of $28.00 per pair of samples, 1A and 1B in accordance with PAC sampling plans. CD samples are also billed at the rate of $28.00 per pair of samples. A charge of 1 to 2 hours per analysis will be assessed the applicant for all official and unofficial samples analyzed by the CB Method, depending upon the difficulty of sample preparation.

The bill should be made out to the applicant as shown on the FV-187. The fee bill should list the certificate numbers, the number of analyses, and the fee.

B. Peanut Administrative Committee Billing

The PAC is never shown as the applicant on any certificate. They do pay for the 2A and 2B samples and the 3A and 3B samples, which will be billed at $28.00 per pair.

When sending the bill to PAC, send a copy of each certificate which the bill covers. Send the bill and certificate to the address shown in section IV.A.3.

WEIGHT - VOLUME CHART (WATER SLURRY METHOD)

This chart is for the determination of the amount of water to be added to the sample of ground peanuts to result in 10.0 grams of peanuts in the 50 ml aliquot of methanol-water used in the final determination. This table takes into account the concentrative properties of methanol and water.

Sample Weight (grams)	Volume of Water (mls)
1000	1455
1010	1469
1020	1484
1030	1498
1040	1513
1050	1527
1060	1542
1070	1556
1080	1571
1090	1586
1100	1600
1110	1615
1120	1629
1130	1644
1140	1658
1150	1673
1160	1687
1170	1702
1180	1716
1190	1731
1200	1746

CALCULATION AID

B1 and G1

Standard Spot (ul)	Sample Spot (ul) 1	2	3	5	6.5
0.25	7.5	3.8	2.5	1.5	1.2
0.5	15.0	7.5	5.0	3.0	2.3
0.75	22.5	11.3	7.5	4.5	3.5
1.0	30.0	15.0	10.0	6.0	4.6
1.25	37.5	18.8	12.5	7.5	5.8
1.5	45.0	22.5	15.0	9.0	6.9
1.75	52.5	26.3	17.5	10.5	8.1
2.0	60.0	30.0	20.0	12.0	9.2
2.25	67.5	33.8	22.5	13.5	10.4
2.5	75.0	37.5	25.0	15.0	11.5
2.75	82.5	41.3	27.5	16.5	12.7
3.0	90.0	45.0	30.0	18.0	13.8
3.25	97.5	48.8	32.5	19.5	15.0
3.5	105.0	52.5	35.0	21.0	16.2
3.75	112.5	56.3	37.5	22.5	17.3
4.0	120.0	60.0	40.0	24.0	18.5
4.25	127.5	63.8	42.5	25.5	19.6
4.5	135.0	67.5	45.0	27.0	20.8
4.75	142.5	71.3	47.5	28.5	21.9
5.0	150.0	75.0	50.0	30.0	23.1

NOTE: This chart is for use only when W (the equivalent weight of the peanuts in the sample) is equal to 10.0 grams.

CALCULATION AID

B2 and G2

Standard Spot (ul)	Sample Spot (ul) 1	2	3	5	6.5
0.25	2.3	1.1	0.8	0.5	0.3
0.5	4.5	2.3	1.5	0.9	0.7
0.75	6.8	3.4	2.3	1.4	1.0
1.0	9.0	4.5	3.0	1.8	1.4
1.25	11.3	5.6	3.8	2.3	1.7
1.5	13.5	6.8	4.5	2.7	2.1
1.75	15.8	7.9	5.3	3.2	2.4
2.0	18.0	9.0	6.0	3.6	2.8
2.25	20.3	10.1	6.8	4.1	3.1
2.5	22.5	11.3	7.5	4.5	3.5
2.75	24.8	12.4	8.3	5.0	3.8
3.0	27.0	13.5	9.0	5.4	4.2
3.25	29.3	14.6	9.8	5.9	4.5
3.5	31.5	15.8	10.5	6.3	4.8
3.75	33.8	16.9	11.3	6.8	5.2
4.0	36.0	18.0	12.0	7.2	5.5
4.25	38.3	19.1	12.8	7.7	5.9
4.5	40.5	20.3	13.5	8.1	6.2
4.75	42.8	21.4	14.3	8.6	6.6
5.0	45.0	22.5	15.0	9.0	6.9

NOTE: This chart is for use only when W (the equivalent weight of the peanuts in the sample) is equal to 10.0 grams.